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14. ABSTRACT

Protein tyrosine kinase 6 (PTK6) is a nonmyristoylated intracellular tyrosine kinase that is expressed in the normal prostate and in prostate cancers. We had hypothesized that PTK6 regulates the activities of its nuclear substrate, the KH domain RNA-binding protein SAM68, to control the epithelial mesenchymal transition (EMT) in prostate cancer. However, during the course of our studies, we determined that PTK6 membrane-associated functions, not nuclear functions, regulate the EMT. We found that while most PTK6 protein is located within the cytoplasm, the pool of active PTK6 is membrane-associated in prostate cancer cells. We showed that membrane-targeted activated PTK6 induces formation of peripheral adhesion complexes accompanied by decreased E-cadherin expression and increased expression of mesenchymal markers, hallmarks of the EMT. Membrane-targeted active PTK6 promoted the epithelial mesenchymal transition in prostate cancer cells, partially through enhancing AKT activation, and increasing anchorage independent growth and cell migration. Our analysis of Oncomine data indicated that high levels of *PTK6* predict poor prognosis for prostate cancer patients. Our findings identify PTK6 as a new candidate therapeutic target in metastatic prostate cancer.

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INTRODUCTION

Prostate cancer is the second leading cause of cancer deaths in men, and lethality of this disease is correlated with the metastasis of the primary tumor. The conversion of epithelial cells into mesenchymal cells that display enhanced migratory and invasion properties has been termed the epithelial-to-mesenchymal transition (EMT). The EMT leads to de-differentiation of epithelial cells, loss of adhesive constraints, and enhanced motility and invasion that are associated with increased tumor grade and metastases (8). Elevated expression and/or activation of tyrosine kinases are often associated with the epithelial-mesenchymal transition (EMT), in which loss of the epithelial marker E-cadherin and elevation of the mesenchymal marker vimentin are observed.

Protein tyrosine kinase 6 (PTK6; also referred to as BRK) is a nonmyristoylated intracellular tyrosine kinase that is evolutionarily related to SRC kinases. Unlike SRC-family members, PTK6 lacks an amino-terminal SH4 domain that promotes lipid modification and membrane association (7). Absence of palmitoylation and/or myristoylation facilitates flexibility in its intracellular localization. The intracellular localization of PTK6 may have a profound impact on signaling, due to its differential access to substrates and associated proteins in different cellular compartments (6, 9, 15, 16).

Sam68, a KH domain RNA-binding protein that belongs to the Signal Transduction and Activation of RNA (STAR) family, is a PTK6 substrate. Sam68 regulates alternative splicing of a number of genes that contribute to prostate cancer (12). Increased expression of Sam68 has been reported in prostate cancers (5). Nuclear PTK6 can phosphorylate Sam68 on several tyrosine residues (2). Sam68 was shown to regulate RNA splicing events that control the EMT (10). In our exploratory grant proposal, we hypothesized that PTK6 regulates the EMT through its ability to modulate its nuclear substrate Sam68 prostate cancer cells. However, during the course of our studies, we found that only membrane-associated PTK6 is active and able to promote the EMT. Membrane-associated PTK6 cannot regulate Sam68 nuclear functions and RNA splicing. We found that membrane-targeted active PTK6 promotes the EMT at least partially through regulation of AKT and p130CAS. Through analyzing data available in Oncomine, we determined that high levels of PTK6 correlate with poor prognosis and reduced E-cadherin expression, indicative of the EMT, in prostate cancer patients.

BODY

KEY RESEARCH ACCOMPLISHMENTS

We completed the following tasks outlined in the Statement of Work:

- Introduce Myc-tagged Sam68 and different PTK6 expression constructs (untargeted, NLS-tagged, and Myr/Palm tagged wild type, constitutively active, kinase dead) or empty vector control into prostate cell lines.

- Isolate protein in Triton-X 100 buffer and RIPA buffer and mRNA.

- Examine expression and intracellular localization of ectopic PTK6, and Sam68 using immunoblotting and total cell lysates or lysates from fractionated cells.

- Perform immunoprecipitations and coimmunoprecipitations to exam protein phosphorylation (PY immunoblotting) and protein-protein associations.

- Perform protein (immunoblotting) and gene expression (qRT-PCR) studies directed at examining EMT marker expression.

- Since we determined that membrane associated PTK6, not nuclear PTK6, was important for induction of the the EMT, we examined the impact of introducing siRNAs against PTK6, and its membrane substrates AKT and p130CAS (instead of SAM68) on induction of the EMT. We studied the impact of siRNA knockdown of AKT and p130CAS on PTK6 mediated regulation of the EMT, and examined expression of EMT markers.

Two PCRP Focus Areas were addressed by the proposal. 1) Therapy: Identification of new targets, pathways, and therapeutic modalities; and 2) Tumor Biology and Immunology. Our studies highlighted new roles for PTK6 in driving the EMT in prostate cancer.

REPORTABLE OUTCOMES

Note: Data described below are presented in the manuscript by Zheng et al. that is included in the appendix: Zheng Y, Wang Z, Bie W, Brauer PM, Perez-White BE, Li J, Nogueira V, Raychaudhuri P, Hay N, Tonetti D, Macias V, Kajdacsy-Balla A, Tyner AL. 2013. PTK6 activation at the membrane regulates epithelial-mesenchymal transition in prostate cancer. *Cancer Research.* **73**(17):5426-37. PMCID: PMC3766391. This is cited reference number 17.

Active PTK6 is membrane associated (Fig. 1 A and B, Reference 17, Zheng et al. 2013 in the appendix)

Phosphorylation of PTK6 at tyrosine residue 342 within its activation loop promotes activation (3). We examined the localization of total PTK6 and active PTK6, phosphorylated on tyrosine residue 342 (PY342), in three prostate epithelial cell lines PC3, DU145 (metastatic) and BPH1 (benign hyperplasia) (1). Cells were fractionated into cytoplasmic, membrane/organelle and nuclear compartments. In all cell lines, total PTK6 was primarily localized in the cytoplasm. However, immunoblotting for PY342 revealed that active PTK6 was localized at the membrane. We did not detect active nuclear PTK6 in prostate cell lines, where Sam68 is located.

Membrane-associated PTK6 induces changes in cell morphology and the EMT (Fig. 1 C - E and Fig. 2 in Reference 17, Zheng et al. 2013 in the appendix)

To explore functions of membrane associated active PTK6, prostate cancer cell lines stably expressing different expression constructs including empty vector, untargeted PTK6, NLS-tagged PTK6, and membrane targeted Palm-tagged wild type, constitutively active, kinase dead PTK6. Palm-PTK6-YF contains dual fatty acylation sites for palmitoylation/myristoylation from the SRC-family kinase LYN at the amino terminus for membrane association (referred to here as Palm), and mutation of the negative regulatory tyrosine at position 447 to phenylalanine (YF) (9). Compared with vector control cells, both PC3 and BPH1 cells expressing Palm-PTK6-YF underwent profound morphological changes, which include a ruffled membrane, formation of peripheral adhesions complexes, and fewer cell-cell contacts, characteristic of the EMT. These morphological changes were not observed in cells expressing either untargeted or nuclear-targeted or kinase dead PTK6.

Loss of E-cadherin is one of the hallmarks of EMT (8). Reduced E-cadherin levels were detected in the presence of Palm-PTK6-YF in PC3 cells. We examined expression of other EMT markers and found that levels of vimentin and the E-cadherin transcriptional repressor ZEB1 are increased in cells expressing Palm-PTK6-YF. Expression of Palm-PTK6-YF decreased membrane association of E-cadherin, increased ZEB1 in the nucleus, and increased vimentin in the cytoplasm and membrane. Levels of mRNAs encoding EMT markers was measured by either quantitative real-time PCR or semi-quantitative PCR. Consistent with protein levels, expression of E-cadherin mRNA was decreased, while levels of mRNAs encoding the mesenchymal intermediate filament protein vimentin, and transcriptional repressors of E-cadherin SLUG, Twist and ZEB1 mRNAs were increased.

AKT participates in PTK6-mediated induction of the EMT (Fig. 3 in Reference 17, Zheng et al. 2013 in the appendix)

AKT was reported to regulate the EMT in carcinoma cell lines (4). We observed increased AKT activation in response to FBS stimulation in Palm-PTK6-YF expressing cells and examined if AKT and downstream signaling are involved in the PTK6 mediated EMT. AKT is a PTK6 associated protein and substrate (11). Phosphorylation of AKT at Thr308 and Ser473, which is required for its activation, was increased in PC3 cells expressing Palm-PTK6-YF relative to total AKT. This was accompanied by increased inhibitory phosphorylation of GSK3β, a direct target of AKT. AKT regulates the SNAIL family member

SLUG (SNAI2), a transcription factor that represses E-cadherin (14). We saw increased and nuclear localization of SLUG in Palm-PTK6-YF expressing PC3 cells.

To test if AKT activation is required for the PTK6 induced EMT, siRNAs were used to knockdown endogenous AKT in PC3 cells. We found that AKT knockdown can partially reverse the PTK6 induced EMT, but other mechanisms are also involved. We also used siRNAs to knock down the scaffold protein p130CAS, a PTK6 substrate, which is crucial for AKT activation (15). Following knockdown of p130CAS, AKT activity was reduced, while total AKT levels were not changed. As in the AKT siRNA experiment, reduction of AKT activation through knockdown of p130CAS only partially rescued EMT induced by Palm-PTK6-YF.

CONCLUSION

The active form of PTK6 is at the plasma membrane in prostate tumor cells not in the nucleus were it might regulate the splicing factor Sam68. Activation of PTK6 at the plasma membrane induces profound morphological changes in prostate cell lines and promotes the EMT. PTK6 at least partially regulates the EMT through its substrates p130CAS and AKT. The EMT signature contributes to metastasis, drug resistance, and poor prognosis in prostate cancers (13). We also detected active PTK6 at the membrane in human tumor samples and determined that higher levels of PTK6 expression correlated with poor prognosis for prostate cancer patients (see Fig. 7 in Reference 17, Zheng et al. 2013 in the appendix). Our studies define PTK6 as a new candidate therapeutic target in prostate cancer.

Project Bibliography:

Two manuscripts that cite DOD support were published and are included in the Appendix.

Zheng Y, Wang Z, Bie W, Brauer PM, Perez-White BE, Li J, Nogueira V, Raychaudhuri P, Hay N, Tonetti D, Macias V, Kajdacsy-Balla A, Tyner AL. 2013. PTK6 activation at the membrane regulates epithelial-mesenchymal transition in prostate cancer. *Cancer Research.* **73**(17):5426-37. PMCID: PMC3766391

Zheng Y, Tyner AL. 2013. Context-specific protein tyrosine kinase 6 (PTK6) signalling in prostate cancer. *Eur J Clin Invest.* **43**(4):397-404.

Data were presented at the annual American Association for Cancer Research meeting in 2013:

Tyner A. L. and Y. Zheng. Protein Tyrosine Kinase 6 promotes peripheral adhesion complex formation, cell migration, and the epithelial mesenchymal transition in prostate cancer. AACR annual meeting April 2013, *Cancer Research*: April 2013.

Personnel receiving pay from the award:

Wenjun Bie

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APPENDICES

Two manuscripts that cite DOD support are attached.

Zheng Y, Wang Z, Bie W, Brauer PM, Perez-White BE, Li J, Nogueira V, Raychaudhuri P, Hay N, Tonetti D, Macias V, Kajdacsy-Balla A, Tyner AL. 2013. PTK6 activation at the membrane regulates epithelial-mesenchymal transition in prostate cancer. *Cancer Research.* **73**(17):5426-37. PMCID: PMC3766391

Zheng Y, Tyner AL. 2013. Context-specific protein tyrosine kinase 6 (PTK6) signalling in prostate cancer. *Eur J Clin Invest.* **43**(4):397-404.

Cancer Research

PTK6 Activation at the Membrane Regulates Epithelial–Mesenchymal Transition in Prostate Cancer

Yu Zheng[1], Zebin Wang[1], Wenjun Bie[1], Patrick M. Brauer[1], Bethany E. Perez White[2], Jing Li[1], Veronique Nogueira[1], Pradip Raychaudhuri[1,4], Nissim Hay[1,4], Debra A. Tonetti[2], Virgilia Macias[3], André Kajdacsy-Balla[3], and Angela L. Tyner[1]

Abstract

The intracellular tyrosine kinase protein tyrosine kinase 6 (PTK6) lacks a membrane-targeting SH4 domain and localizes to the nuclei of normal prostate epithelial cells. However, PTK6 translocates from the nucleus to the cytoplasm in human prostate tumor cells. Here, we show that while PTK6 is located primarily within the cytoplasm, the pool of active PTK6 in prostate cancer cells localizes to membranes. Ectopic expression of membrane-targeted active PTK6 promoted epithelial–mesenchymal transition in part by enhancing activation of AKT, thereby stimulating cancer cell migration and metastases in xenograft models of prostate cancer. Conversely, siRNA-mediated silencing of endogenous PTK6 promoted an epithelial phenotype and impaired tumor xenograft growth. In mice, PTEN deficiency caused endogenous active PTK6 to localize at membranes in association with decreased E-cadherin expression. Active PTK6 was detected at membranes in some high-grade human prostate tumors, and PTK6 and E-cadherin expression levels were inversely correlated in human prostate cancers. In addition, high levels of PTK6 expression predicted poor prognosis in patients with prostate cancer. Our findings reveal novel functions for PTK6 in the pathophysiology of prostate cancer, and they define this kinase as a candidate therapeutic target. *Cancer Res; 73(17); 5426–37. ©2013 AACR.*

Introduction

Prostate cancer is the second most common cancer and second leading cause of cancer-related death in American men (1). Most prostate cancer-related deaths are due to advanced metastatic disease, resulting from lymphatic, blood, or contiguous local spread. Tumors of the prostate originate from epithelial cells and there is a clinical correlation between the degree of differentiation and clinical outcomes.

Protein tyrosine kinase 6 (PTK6, also known as BRK or Sik) is a SRC-related intracellular tyrosine kinase that is expressed in epithelial cells. Unlike SRC-family members, PTK6 lacks an amino-terminal SH4 domain that promotes lipid modification and membrane association (2). Absence of palmitoylation and/or myristoylation facilitates flexibility in its intracellular localization. The intracellular localization of PTK6 may have a profound impact on signaling, due to its differential access to substrates and associated proteins in different cellular compartments (3–5). Currently, the prostate provides the only known physiologically relevant example of PTK6 relocalization *in vivo*. PTK6 is primarily nuclear in epithelial cells of the normal human prostate, but nuclear localization is lost in prostate cancer (6). Cytoplasmic retention of PTK6 promoted growth of the PC3 prostate cancer cell line, whereas expression of nuclear-targeted PTK6 significantly decreased cell proliferation (7).

Expression of PTK6 is elevated in several epithelial-derived cancers such as breast, colon, head and neck, melanoma, and ovarian cancer (reviewed in refs. 8, 9). Increased levels of PTK6 mRNA were detected in metastatic human prostate cancer samples, suggesting a role for PTK6 in prostate tumor metastasis (5). PTK6 promotes cancer cell proliferation, migration, and survival through activating oncogenic signaling pathways involving AKT, Paxillin, p190RhoGAP, p130CAS, STAT3, STAT5b, EGF receptor (EGFR), HER2, MET, and insulin-like growth factor-I receptor (IGF-IR; reviewed in refs. 8, 9). PTK6 directly phosphorylates and promotes AKT activation in response to EGF in BPH1 cells (10). It directly phosphorylates the CRK-associated substrate p130CAS, leading to formation of peripheral adhesion complexes and enhanced cell migration in PC3 cells (5). Recently, PTK6 was also shown to phosphorylate and activate focal adhesion kinase (FAK) to promote resistance to anoikis (11).

Elevated expression and/or activation of tyrosine kinases are often associated with the epithelial–mesenchymal transition (EMT), in which loss of the epithelial marker E-cadherin and elevation of the mesenchymal marker vimentin are observed (12). Activated SRC induces disorganization of E-cadherin–dependent cell–cell contacts and vimentin expression in the KM12C colon cancer cell line. Deregulation of E-cadherin and formation of peripheral adhesions induced by active SRC kinase

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relies on integrin, FAK, and extracellular signal–regulated kinase (ERK)1/2 signaling cascades (13, 14). Recent studies indicate that the EMT of tumor cells is also coupled with increased cell survival and drug resistance (reviewed in ref. 15).

We report endogenous PTK6 activation at the membrane in prostate epithelial cell lines, *Pten*-null mice, and human prostate tumors. We found that membrane-targeted active PTK6 causes a cell-scattering phenotype in PC3 cells and promotes the EMT, cell migration, and invasion. This is achieved at least partially through increased activation of AKT. Knockdown of PTK6 in PC3 cells promotes an epithelial phenotype and dramatically reduces metastases *in vivo*. In contrast, activation of PTK6 at the plasma membrane is associated with deregulation of E-cadherin in mouse and human prostates. High levels of PTK6 also predict a poor prognosis for patients. Our studies show a novel role for PTK6 in the EMT and suggest that PTK6 can be a target for treating metastatic prostate cancer.

Materials and Methods

Antibodies

Anti-human PTK6 (C-18, G-6), mouse PTK6 (C-17), SP1 (PEP2), E-cadherin (H-108), ZEB1 (H-102), p63 (4A4), and anti-phospho-tyrosine (PY20) antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-tyrosine (clone 4G10) and anti-P-PTK6 (Tyr342) antibodies were purchased from Millipore. Antibodies directed against AKT, P-AKT (Thr308), P-AKT (Ser473), P-GSK3β (Ser9), SLUG, and Myc-tag (9B11) were obtained from Cell Signaling Technology. Antibodies directed against β-catenin and BrdUrd were obtained from BD Pharmingen. Anti-β-actin (AC-15) and vimentin antibodies were purchased from Sigma-Aldrich. Anti-CK5 antibodies were a gift of Dr. G. Paolo Dotto (University of Lausanne, Lausanne, Switzerland). Anti-CK8 and Ki67 antibodies were purchased from Abcam. Donkey anti-rabbit or sheep anti-mouse antibodies conjugated to horseradish peroxidase were used as secondary antibodies (Amersham Biosciences) and detected by chemiluminescence with SuperSignal West Dura extended duration substrate from Pierce.

Plasmids and siRNAs

The Myc-tagged Palm-PTK6-YF construct in the pBABE-puro vector has been described previously (10). The siRNAs (Dharmacon) targeting p130CAS: 5′-GGTCGACAGTGGTGTG-TAT-3′ (5) and AKT: 5′-TGCCCTTCTACAACCAGGATT-3′ (16) were previously reported. Dicer-substrate siRNAs against PTK6 were purchased from the Integrated DNA Technologies predesigned DsiRNA library. The sequence for Dsi-PTK6 is 5′-AGGTTCACAAATGTGGAGTGTCTGC-3′.

Cell culture and fractionation

The human prostate cancer cell lines PC3 [American Type Culture Collection (ATCC); CRL-1435] and DU145 (ATCC; HTB-81) were certified by ATCC and cultured according to the ATCC guidelines. The benign prostatic hyperplasia epithelial cell line BPH-1 (kindly provided by Simon Hayward, Vanderbilt University, Nashville, TN; ref. 17) was cultured in RPMI-1640 containing 5% FBS. No additional authentication of cell lines was conducted. Cell fractionations were carried out using the ProteoExtract Subcellular Proteome Extraction Kit (EMD Millipore) according to the manufacturer's instructions. The method used for preparation of total cell lysates has been described previously (10).

Retrovirus production and transduction

pBABE-puro plasmids were transfected into Phoenix-Ampho cells using Lipofectamine 2000 (Invitrogen). Retrovirus was collected 48 and 72 hours later. PC3 and BPH1 cells were infected with retrovirus at a multiplicity of infection (MOI) of 100 for 24 hours. Stable cell pools were selected in growth medium containing 2 μg/mL puromycin for 1 week.

Primers and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). After DNase I digestion (Promega), 500 ng of RNA was used to generate cDNA using a cDNA synthesis kit (Bio-Rad). Real-time (RT)-PCR was conducted using the following mixture: 1× iQ SYBR Green Supermix (Bio-Rad), 100 nmol/L of each primers, and 1 μL of cDNA in a 25 μL total volume. Reactions were amplified and analyzed in triplicate using a MyiQ single-color RT-PCR detection system (Bio-Rad). The following primers were used: human cyclophilin: (forward) 5′-GCAGACAAG-GTCCCAAAGACAG-3′ and (reverse) 5′-CACCCTGACACAT-AAACCCTGG-3′; human E-cadherin: (forward) 5′-ATGCT-GATGCCCCCAATACC-3′ and (reverse) 5′-TCCAAGCCCTT-TGCTGTTTTC-3′; human vimentin: (forward) 5′-TTGACAA-TGCGTCTCTGGCAC-3′ and (reverse) 5′-CCTGGATTTCCT-CTTCGTGGAG-3′; human ZEB1: (forward) 5′-AACGCTTTT-CCCATTCTGGC-3′ and (reverse) 5′-GAGATGTCTTGAGTCC-TGTTCTTGG-3′; human SLUG: (forward) 5′-GCTCAGAAAGC-CCCATTAGTGATG-3′ and (reverse) 5′-GCCAGCCCAGAAA-AAGTTGAATAG-3′; human Twist: (forward) 5′-GTCCGC-AGTCTTACGAGGAG-3′ and (reverse) 5′-CCAGCTTGAGGG-TCTGAATC-3′; and human PTK6: (forward) 5′-GCTATGTG-CCCCACAACTACC-3′ and (reverse) 5′-CCTGCAGAGCGT-GAACTCC-3′.

Proliferation, colony formation, and soft agar assays

For proliferation assays, subconfluent cells were seeded in triplicate for each time point at a density of 2×10^3 cells per well of 48-well plates. The fold increase in cell number was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega). For colony formation assays, cells were seeded in triplicate at a density of 1×10^3 cells per well of 6-well plates 24 hours after transfection, and grown for 14 days before fixing and staining with crystal violet (Sigma-Aldrich). For soft agar assays, 1.5×10^3 cells were seeded in triplicate on the top layer of 6-well plates, which contained 0.35% agar in growth medium containing 10% FBS. The bottom layer of soft agar contained 0.7% agar in growth medium containing 10% FBS. Cells were fed twice a week, and colonies were counted at 3 weeks after plating.

Migration and invasion chamber assays

For migration assays, cells were transfected with siRNAs for 24 hours if needed and then serum-starved for another 24 hours. A total of 5×10^4 cells were plated in the top chamber of

a Transwell (24-well insert; pore size, 8 μm; Corning) and incubated with 1% FBS containing medium. Twenty percent FBS-containing medium was added to the lower chamber as a chemoattractant. After 18 hours, cells that did not migrate through the pores were removed by a cotton swab, and the cells on the lower surface of the membrane were stained by crystal violet. BD BioCoat Matrigel Invasion Chambers (BD Pharmingen) were used for invasion assays, which were conducted in a similar way to migration assays, except that 50 ng/mL HGF was used as a chemoattractant and the incubation time was 24 hours. Images were taken under the phase-contrast microscope using ×10 magnification.

Immunostaining

Cells were washed with PBS, fixed in Carnoy's solution (6:3:1 ethanol:chloroform:acetic acid), then blocked with 3% bovine serum albumin for 1 hour, and incubated with primary antibodies overnight. Fluorescein isothiocyanate (FITC)–conjugated anti-mouse secondary antibodies (Sigma-Aldrich) were used to detect primary antibodies made in mouse (green), and biotinylated anti-rabbit secondary antibodies (Vector Laboratories) were used and then incubated with rhodamine-conjugated avidin to detect primary antibodies made in rabbit (red). Slides were mounted with Vectashield fluorescent mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

For staining of prostate tissues and tumors, antigen retrieval was conducted in 10 mmol/L sodium citrate buffer on a hot plate at a temperature above 90°C for 20 minutes. Immunohistochemistry was conducted using the VECTASTAIN Elite ABC Kit [rabbit immunoglobulin G (IgG)] or the mouse on mouse (M.O.M.) kit as per the manufacturer's instructions (Vector Laboratories). Reactions were visualized with FITC or rhodamine-conjugated avidin, and slides were mounted in Vectashield fluorescent mount media containing DAPI, or with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) and counterstained with hematoxylin (Vector Laboratories). Staining controls were conducted with normal rabbit or mouse IgG.

Xenograft and murine prostate cancer models

To monitor metastases *in vivo*, pFU-L2G, which expresses optimized luciferase (L2) and GFP (G; ref. 18), was introduced into PC3 cells. Cells, selected for GFP expression, were transfected with PTK6 siRNA or control siRNA twice before intravenous injection into 6-week-old male SCID (*IcrTac:ICR-Prkdc*^{scid}; Taconic) mice. Tumor growth and metastases were monitored weekly following injection of D-luciferin using the Xenogen IVIS Spectrum *in vivo* imaging system (Caliper Life Sciences, Inc.). Alternatively, cells were introduced by intracardiac injection and mice were sacrificed at 10 weeks, and internal organs were formalin-fixed and paraffin-embedded. Generation and characterization of the PB-Cre4 and *Pten*$^{flox/flox}$ mice have been described previously (19). C57BL/6J PB-Cre4 *Pten*$^{flox/flox}$ mice were sacrificed at 6 months of age and prostates were formalin-fixed and paraffin-embedded. All mouse experiments were reviewed and approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

Statistical analysis

Datasets containing 363 and 140 primary prostate cancer samples and the patient information were extracted from the Oncomine database (Compendia Bioscience). These include the Setlur Prostate Dataset, National Center for Biotechnology Information (NCBI) dataset GSE8402 (20), and the Taylor prostate dataset, NCBI dataset GSE21035 (21). Patients were categorized into "*PTK6* high," "*PTK6* medium," and "*PTK6* low" groups according to their *PTK6* RNA expression levels. The *PTK6* high group represents the top 10%, whereas the *PTK6* low group represents the bottom 25% of the patients according to PTK6 RNA levels. The *PTK6* medium group represents the remaining patients with intermediate *PTK6* expression levels. The survival curve or recurrence rate was estimated using the Kaplan–Meier method and the differences among three groups was tested using the log-rank test. The analysis was conducted using SAS 9.2. *PTK6* and E-cadherin mRNA levels were analyzed in a NCBI human genome microarray dataset GDS2545, which contains 171 human prostate samples including normal prostate tissue, normal tissue adjacent to the primary tumor, primary tumor, and metastatic tumors. Results are shown as the mean ± SE. A linear regression model is set up using E-cadherin mRNA as a dependent variable and PTK6 as an independent variable. For all the other cell studies, data represent the mean of at least 3 independent experiments ± SD. *P* values were determined using the one-tailed Student *t* test (Microsoft Excel 2010) and two-sided Fisher exact test (GraphPad Prism 5). A difference was considered statistically significant if the *P* value was equal to or less than 0.05.

Results

Membrane-targeted PTK6 causes a cell-scattering phenotype in prostate epithelial cells

PTK6 relocalizes from the nucleus to the cytoplasm in prostate epithelial tumor cells, as human prostate cancer progresses (6). Phosphorylation of PTK6 at tyrosine residue 342 within its activation loop promotes activation (22). We examined the localization of total PTK6 and active PTK6, phosphorylated on tyrosine residue 342 (PY342), in three prostate epithelial cell lines PC3, DU145 (metastatic), and BPH1 (benign hyperplasia; ref. 17). Cells were fractionated into cytoplasmic, membrane/organelle, and nuclear compartments. In all three cell lines, total PTK6 is primarily localized in the cytoplasm (Fig. 1A). However, immunoblotting for PY342 revealed that active PTK6 is localized at the membrane (Fig. 1A).

To explore functions of membrane-associated active PTK6, PC3 and BPH1 cell lines stably expressing membrane-targeted active PTK6 (Palm-PTK6-YF) were generated. Palm-PTK6-YF contains dual fatty acylation sites for palmitoylation/myristoylation from the SRC-family kinase LYN at the amino terminus for membrane association (referred to here as Palm), and mutation of the negative regulatory tyrosine at position 447 to phenylalanine (YF; ref. 4). Ectopic expression of Palm-PTK6-YF was confirmed by immunoblotting (Fig. 1B). Compared with vector control cells, both PC3 and BPH1 cells expressing Palm-PTK6-YF undergo profound morphologic changes, which include a ruffled membrane and fewer cell–cell contacts (Fig. 1C). The ruffled membrane suggests the

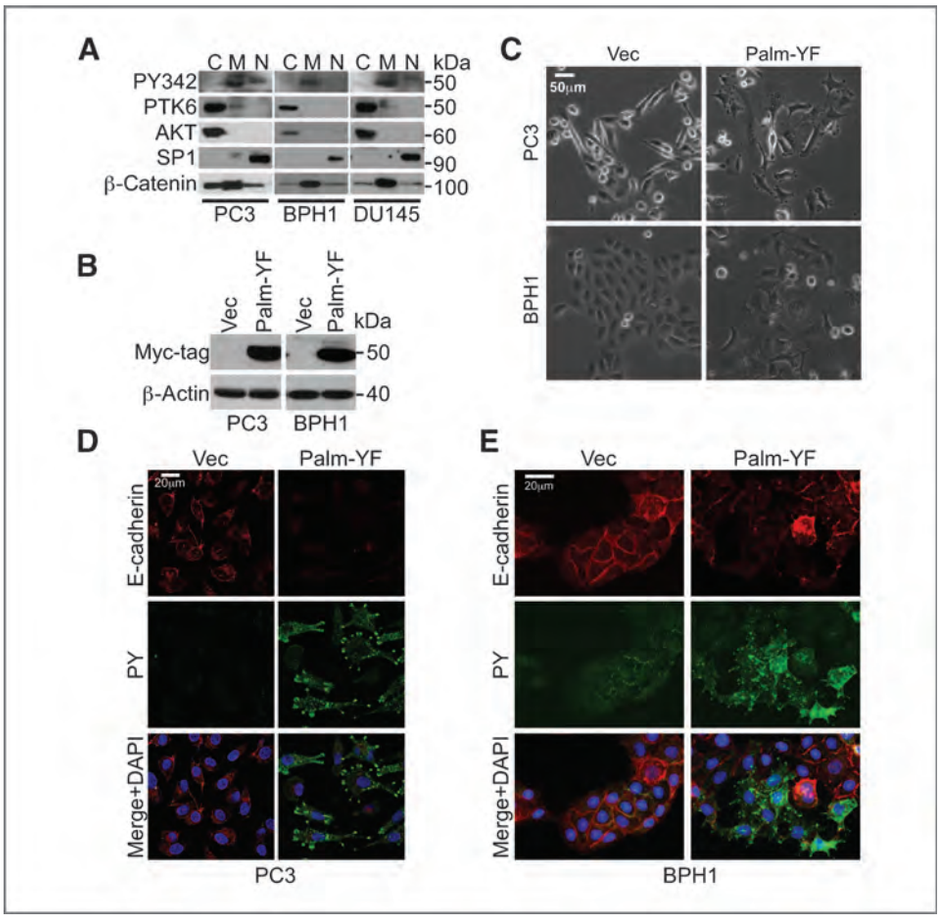

Figure 1. Expression of Palm-PTK6-YF induced a cell-scattering phenotype in BPH1 and PC3 cells. A, the membrane pool of PTK6 is the active pool. PC3, DU145, and BPH1 cells were fractionated into three cellular compartments including cytoplasm, membrane/organelle, and nucleus. Immunoblot analysis was conducted with anti-P-PTK6 (PY342), PTK6, AKT, SP1, and β-catenin antibodies. AKT, SP1, and β-catenin localization were examined as controls for fractionation. Although the majority of total PTK6 protein is cytoplasmic, the active pool (PY342) is membrane associated. B, Palm-PTK6-YF was stably expressed in PC3 and BPH1 cells. Immunoblot analysis was conducted using anti-Myc-tag and β-actin antibodies. C, cells expressing Palm-PTK6-YF (B) show the cell-scattering phenotype. Phase-contrast images of PC3 and BPH1 cells stably expressing Palm-PTK6-YF or vector are shown. Scale bar, 50 μm. D, loss of E-cadherin at the membrane in PC3 cells stably expressing Palm-PTK6-YF. Cells were costained with anti-E-cadherin and phospho-tyrosine (PY) antibodies, and counterstained with DAPI (blue). Scale bar, 20 μm. E, BPH1 cells that form peripheral adhesion complexes show deregulated E-cadherin at the cell membrane. Cells were costained with anti-E-cadherin and phospho-tyrosine antibodies and counterstained with DAPI (blue). Scale bar, 20 μm.

formation of PTK6-induced peripheral adhesion complexes as reported previously (5). Formation of these peripheral adhesion complexes was dependent upon PTK6 kinase activity and did not form in cells expressing membrane-targeted kinase defective PTK6 (5).

A cell-scattering phenotype is often coupled with the EMT (12). Because both BPH1 and PC3 cells express E-cadherin, we examined whether E-cadherin expression and localization are altered by Palm-PTK6-YF expression. In both PC3 and BPH1 cells, expression of Palm-PTK6-YF led to a reduction in E-cadherin at the plasma membrane that was accompanied by activation of phospho-tyrosine signaling in peripheral adhesion complexes (Fig. 1D and E). BPH1 cells that do not form peripheral adhesion complexes with high levels of phospho-tyrosine still contain E-cadherin at cell–cell contacts (Fig. 1E), suggesting that phospho-tyrosine signaling is involved in deregulating E-cadherin.

Active PTK6 at the plasma membrane promotes the EMT

Loss of E-cadherin is one of the hallmarks of EMT (12). Reduced E-cadherin levels were detected in the presence of Palm-PTK6-YF in PC3 cells (Fig. 2A). We examined expression of other EMT markers and found that levels of vimentin and the E-cadherin transcriptional repressor ZEB1 are increased in cells expressing Palm-PTK6-YF (Fig. 2A). Following cell fractionation, we found that ectopic expression of Palm-PTK6-YF largely increases the pool of active PTK6 at the membrane (Fig. 2B; PY342). Endogenous membrane-associated phospho-PTK6 is the main band detected by immunoblotting of control PC3 cell lysates with anti-PY342 (Fig. 2C, vector lanes, arrowhead). Ectopic transfected Palm-PTK6 migrates slightly above the endogenous band (Fig. 2C; Palm-YF). Expression of Palm-PTK6-YF leads to decreased membrane association of E-cadherin, increased ZEB1 in the nucleus, and increased vimentin in the cytoplasm and membrane (Fig. 2B). Levels of mRNAs

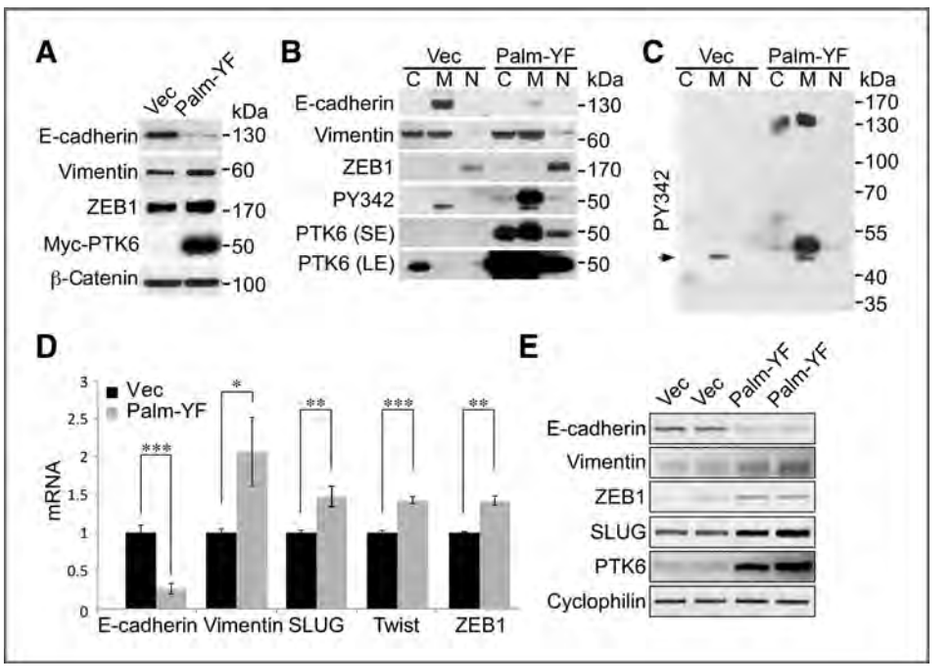

Figure 2. Active PTK6 at the plasma membrane promotes EMT in prostate tumor cells. A, immunoblot analysis of total cell lysates of PC3 cells stably expressing Palm-PTK6-YF or vector was conducted using anti-E-cadherin, vimentin, ZEB1, Myc-tag, and β-catenin antibodies. Expression of β-catenin does not change in cells expressing PTK6-Palm-YF (4) and it was used as a loading control. B, a subcellular fractionation assay was conducted using PC3 cells expressing Palm-PTK6-YF or vector, and immunoblot analysis was conducted using anti-E-cadherin, vimentin, ZEB1, P-PTK6 (PY342), and PTK6 antibodies. Both short (SE) and long (LE) exposures of PTK6 immunoblot are shown. C, an uncropped blot is presented to show specificity of the PY342 antibody. An arrowhead points to endogenous active PTK6 localized at the membrane in PC3 cells (Vec). Ectopic Palm-PTK6-YF runs slightly above the endogenous band in transfected cells (Palm-YF). D, mRNA levels of EMT markers are deregulated in Palm-PTK6-YF–expressing PC3 cells. qRT-PCR was conducted, and E-cadherin, vimentin, SLUG, Twist, and ZEB1 mRNA levels were normalized to cyclophilin mRNA levels. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. E, semiquantitative PCR was conducted to monitor the change of mRNA levels of EMT markers, including E-cadherin, vimentin, ZEB1, and SLUG. Cyclophilin served as a loading control.

encoding EMT markers were also measured by either quantitative real-time PCR (qRT-PCR) or semiquantitative PCR. Consistent with protein levels, expression of E-cadherin mRNA is decreased, whereas levels of mRNAs encoding the mesenchymal intermediate filament protein vimentin, and transcriptional repressors of E-cadherin SLUG, Twist, and ZEB1 mRNAs are increased (Fig. 2D and E).

AKT participates in PTK6-mediated induction of the EMT

AKT is a crucial regulator of the EMT in squamous cell carcinoma lines (23). Previously, we observed increased AKT activation in response to FBS stimulation in Palm-PTK6-YF–expressing cells (5), and therefore examined whether AKT and downstream signaling are involved in the PTK6-mediated EMT. Phosphorylation of AKT at Thr308 and Ser473, which is required for its activation, is increased in PC3 cells expressing Palm-PTK6-YF relative to total AKT (Fig. 3A). This is accompanied by increased inhibitory phosphorylation of GSK3β, a direct target of AKT (Fig. 3A). AKT has been reported to regulate the SNAIL family member SLUG (SNAI2), a transcription factor that represses E-cadherin (24). We see increased expression (Fig. 3A) and nuclear localization (Fig. 3B) of SLUG in Palm-PTK6-YF–expressing PC3 cells.

To test whether AKT activation is required for the PTK6-induced EMT, we used siRNAs to knockdown endogenous AKT in PC3 cells. Following knockdown of AKT, E-cadherin levels are increased and vimentin levels are decreased in both Palm-PTK6-YF and vector control cells (Fig. 3C). However, expression of E-cadherin in Palm-YF–expressing cells treated with AKT siRNA remains lower than vector control cells treated with scrambled siRNA (Fig. 3C), indicating that AKT knockdown only partially rescues PTK6-induced EMT and that other mechanisms are involved. We also used siRNAs to knockdown the scaffold protein p130CAS, which is crucial for AKT activation (5). Following knockdown of p130CAS, AKT activity is reduced, whereas total AKT levels are not changed (Fig. 3D). Decreased AKT activity is accompanied by decreased GSK3β phosphorylation, increased E-cadherin expression, and decreased vimentin levels in both Palm-PTK6-YF and vector control cells (Fig. 3D). As in the AKT siRNA experiment, reduction of AKT activation through knockdown of p130CAS only partially rescues EMT induced by Palm-PTK6-YF (Fig. 3D).

Palm-PTK6-YF promotes tumorigenicity and invasiveness of PC3 cells

We examined the tumorigenic and invasive ability of PC3 cells stably expressing Palm-PTK6-YF *in vitro* and *in vivo*. Expression of Palm-PTK6-YF promotes anchorage-independent growth of PC3 cells in soft agar (Fig. 4A), while not affecting cell proliferation (data not shown), suggesting that

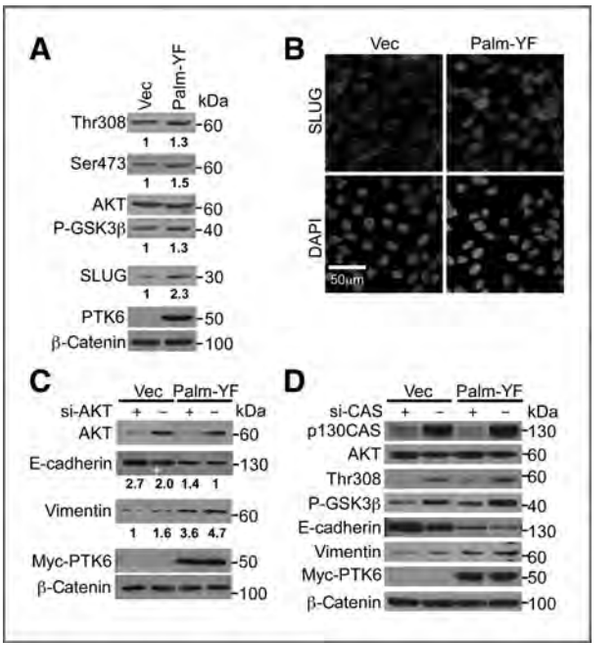

Figure 3. PTK6-mediated EMT occurs partially through increased AKT activity. A, increased AKT signaling in PC3 cells expressing Palm-PTK6-YF. Immunoblot analysis of total cell lysates of PC3 cells stably expressing Palm-PTK6-YF or vector (Vec) was conducted using anti-AKT, P-AKT (Thr308), P-AKT (Ser473), P-GSK3β (Ser9), SLUG PTK6, and β-catenin antibodies. Relative levels of P-AKT, P-GSK3β, and SLUG normalized to β-catenin are indicated below the blots. B, increased nuclear localization of SLUG in PC3 cells stably expressing Palm-PTK6-YF. Cells were stained with anti-SLUG antibody and counterstained with DAPI (blue). Scale bar, 50 μm. C, knockdown of AKT partially rescues Palm-PTK6-YF–induced EMT. PC3 cells expressing Palm-PTK6-YF or vector were transfected with AKT siRNAs or control siRNAs for 3 days. Immunoblotting was conducted with anti-AKT, E-cadherin, vimentin, PTK6, and β-catenin antibodies. Relative levels of E-cadherin and vimentin normalized to the β-catenin loading control are indicated below the blots. D, knockdown of p130CAS partially rescues Palm-PTK6-YF–induced EMT. PC3 cells expressing Palm-PTK6-YF or vector were transfected with p130CAS siRNAs or control siRNAs for 3 days. Immunoblotting was conducted with anti-p130CAS, AKT, P-AKT (Thr308), P-GSK3β (Ser9), E-cadherin, vimentin, Myc-tag, and β-catenin antibodies.

membrane-targeted PTK6 promotes the tumorigenicity of PC3 cells independently of activating cell proliferation pathways. Transwell chamber assays showed that expression of Palm-PTK6-YF promotes cell migration *in vitro* (Fig. 4B). To explore the metastatic characteristics of Palm-PTK6-YF–expressing PC3 cells *in vivo*, intracardiac injection was conducted in 6-week-old severe combined immunodeficient mice (SCID) mice. In the group injected with Palm-PTK6-YF–expressing cells, 3 of 5 mice were dead after 8 weeks, whereas the 2 surviving mice showed dramatic metastases to internal organs including liver, lung, and pancreas after 12 weeks (Fig. 4C). Tumors were visible in lung and liver tissues, and immunohistochemical staining for PTK6 showed membrane association of Palm-PTK6-YF in the tumor tissues, confirming the origin of the tumors (Fig. 4D). In the group injected with control cells, only 1 of 5 mice was found dead after 8 weeks, and no metastases were detected in internal organs in the 4 surviving mice. Using the two-sided Fisher exact test, we determined that the association between Palm-PTK6-YF expression and poor survival outcome is significant ($P < 0.05$). To monitor *in vivo* metastasis, both Palm-PTK6-YF–expressing and control PC3 cells, infected with lentivirus carrying a luciferase gene and a GFP gene, were selected by GFP flow cytometry, and then intravenously injected into SCID mice. At day 0, equal numbers of control vector and Palm-PTK6-YF cells were injected and then traveled to the lungs, as shown in dorsal and ventral views of luciferin-injected mice (Fig. 4E). After 1 week, increased levels of PC3 Palm-PTK6-YF cells were observed, indicating better survival of these tumor cells *in vivo*, leading to increased metastases at day 50 (Fig. 4E). These data show that membrane-targeted active PTK6 promotes the EMT by conferring resistance to anoikis, as well as stimulating anchorage-independent growth and cell migration, resulting in increased metastasis *in vivo*.

PC3 cells are less tumorigenic and invasive after knockdown of PTK6

To determine if endogenous PTK6 participates in the EMT and regulates tumorigenicity of PC3 cells, PTK6 was knocked down using an siRNA-based approach. Knockdown of PTK6 persisted for at least 6 days posttransfection (Fig. 5A). Following PTK6 knockdown, E-cadherin levels increased, whereas vimentin and ZEB1 levels decreased (Fig. 5A). In addition, knockdown of PTK6 resulted in decreased proliferation (Fig. 5B), colony formation (Fig. 5C), and anchorage-independent growth in soft agar (Fig. 5D). After PTK6 knockdown, the ability of PC3 cells to invade through the extracellular matrix layer to the bottom side of the membranes in invasion chamber assays was diminished (Fig. 5E).

We conducted xenograft studies to monitor the impact of PTK6 knockdown on metastasis in SCID mice. Luciferase-expressing PC3 control cells and PTK6 knockdown cells were injected intravenously into SCID mice and monitored *in vivo* following injection with luciferin. Knockdown of PTK6 by siRNA effectively reduced survival and metastasis of PC3 cells, compared with control siRNA-treated cells, which metastasized by day 36 (Fig. 5F).

Activation of endogenous PTK6 at the membrane in *Pten*-null mouse prostates correlates with the EMT

To investigate the significance of PTK6 relocalization *in vivo*, we used a murine prostate cancer model (PB-Cre4, $Pten^{flox/flox}$; ref. 19). Compared with wild-type control mice, disruption of *Pten* led to an abnormally enlarged anterior prostate (AP) at the age of 8 months in male mice (Fig. 6A, white hatch marks). Consistent with previous reports, loss of both *Pten* alleles results in early murine prostatic intraepithelial neoplasia (PIN) formation that can progress to adenocarcinoma (25). Preexisting prostatic ductules and acini in PB-Cre4, $Pten^{flox/flox}$ mice were filled with cells derived from the hyperproliferative epithelium, whereas a single layer of epithelial cells was present in the control mice (Fig. 6B). Knockout of *Pten* and activation of AKT were observed in prostate epithelial cells in PB-Cre4, $Pten^{flox/flox}$ mice (Fig. 6B). As expected, PTK6 was detected within nuclei of normal prostate epithelial cells in wild-type

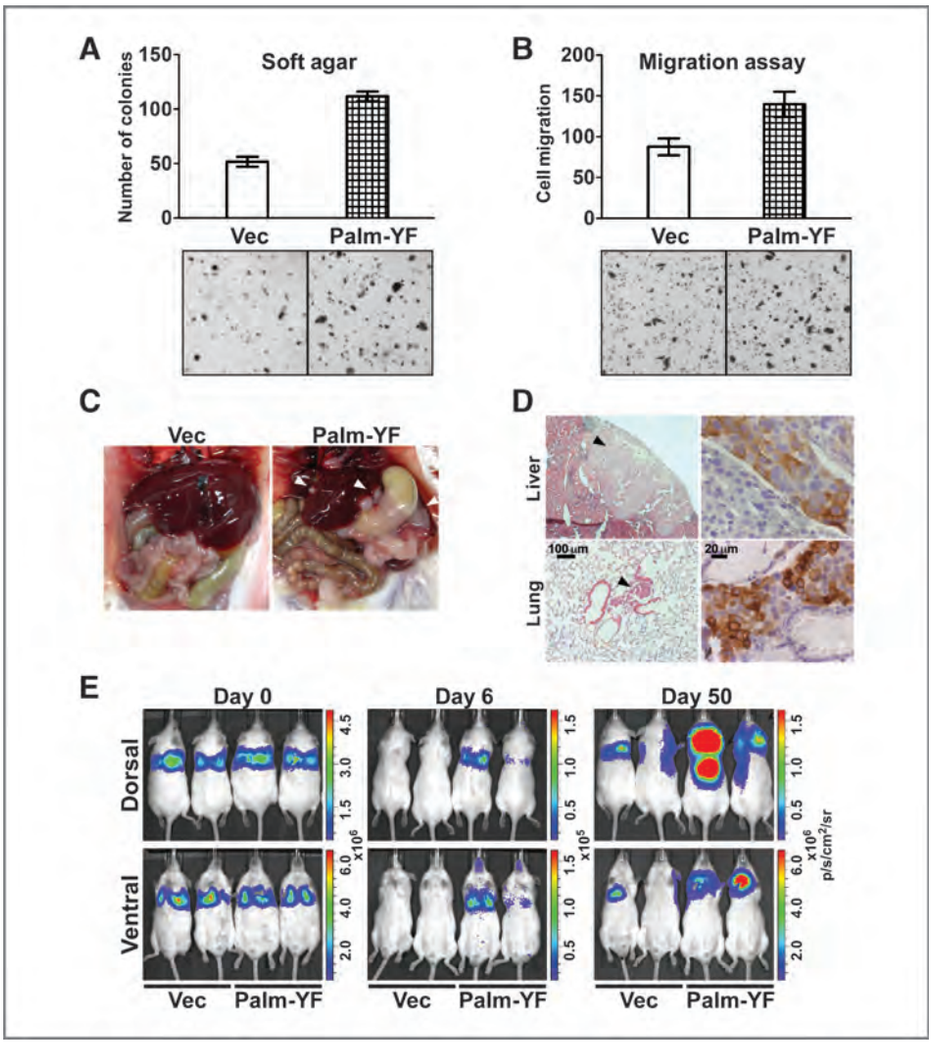

Figure 4. PC3 cells expressing Palm-PTK6-YF are more tumorigenic and invasive. A, Palm-PTK6-YF–expressing cells form increased number of colonies in soft agar. Representative images are shown. B, Palm-PTK6-YF expression promotes cell migration in Transwell chamber assays. Representative images are shown. C, intracardiac injection of PC3 cells expressing Palm-PTK6-YF results in increased metastases to internal organs of immunodeficient SCID mice after 10 weeks. White arrowheads, tumors in liver and pancreas. D, hematoxylin and eosin staining was conducted with lung and liver tumor sections. Black arrowheads, tumors. Scale bar, 100 μm. Immunohistochemistry using anti-PTK6 antibody shows that tumor cells in lung and liver exhibit membrane staining of PTK6 (Palm-PTK6-YF). Scale bar, 20 μm. E, intravenous injection of PC3 cells expressing Palm-PTK6-YF showed increased metastases in SCID mice. Both control and Palm-PTK6-YF–expressing cells stably express luciferase. One million cells were injected intravenously at day 0. Mice were monitored under IVIS spectrum imaging system every week until day 50.

mice, but it was primarily cytoplasmic in the *Pten* null prostate (Fig. 6B; PTK6). Interestingly, in addition to relocalization of PTK6 from nucleus to cytoplasm, we detected significant association of activated endogenous PTK6 phosphorylated at the tyrosine residue 342 with the plasma membrane in the *Pten* null prostates (Fig. 6B; $Pten^{flox/flox}$, PY342). In the wild-type prostate, active PTK6 is largely confined to the nucleus (Fig. 6B; $Pten^{wt/wt}$, PY342).

To determine the lineage of the cells with active membrane associated PTK6, dual immunostaining was conducted using antibodies specific for markers that identify subpopulations of human and murine prostatic epithelial cells (25). Anti-phospho-tyrosine and anti-PTK6 PY342 antibodies recognized the same group of cells with high phospho-tyrosine signaling at the plasma membrane (Fig. 6C, a). An expanded pool of cytokeratin 5 $(CK5)^+$, $p63^+$ (basal cell marker) cells was observed within prostatic ductules upon homozygous *Pten* deletion, consistent with a previous report (25). However, cells with activated PTK6 at the membrane do not express CK5 and p63 (Fig. 6C, b–e), but are CK8 (luminal cell marker)-positive (Fig. 6C, f and g), suggesting they are derived from luminal secretory cells. Most of the phospho-tyrosine–positive cells are not proliferative as evidenced by Ki67 and bromodeoxyuridine (BrdUrd) staining, although there are more proliferating cells in the *Pten* null prostates compared with normal prostate in control mice (Fig. 6C, h–j). Phospho-tyrosine–positive cells are larger than surrounding phospho-tyrosine–negative cells, which led us to examine proteins involved in cell–cell contacts. The cells with activated PTK6 signaling at the plasma membrane show decreased E-cadherin and increased E-cadherin endocytosis (Fig. 6C, k–m). In addition, increased levels of vimentin, a mesenchymal marker, were detected in most of the prostate tumor cells in *Pten* null prostates (Fig. 6C, n and o). These data suggest that cells with high phospho-tyrosine signaling and active PTK6 at the plasma membrane are undergoing the EMT.

High levels of PTK6 predict poor prognosis for prostate cancer patients

To understand the clinical significance of PTK6 in human prostate cancer, a dataset containing 363 primary prostate

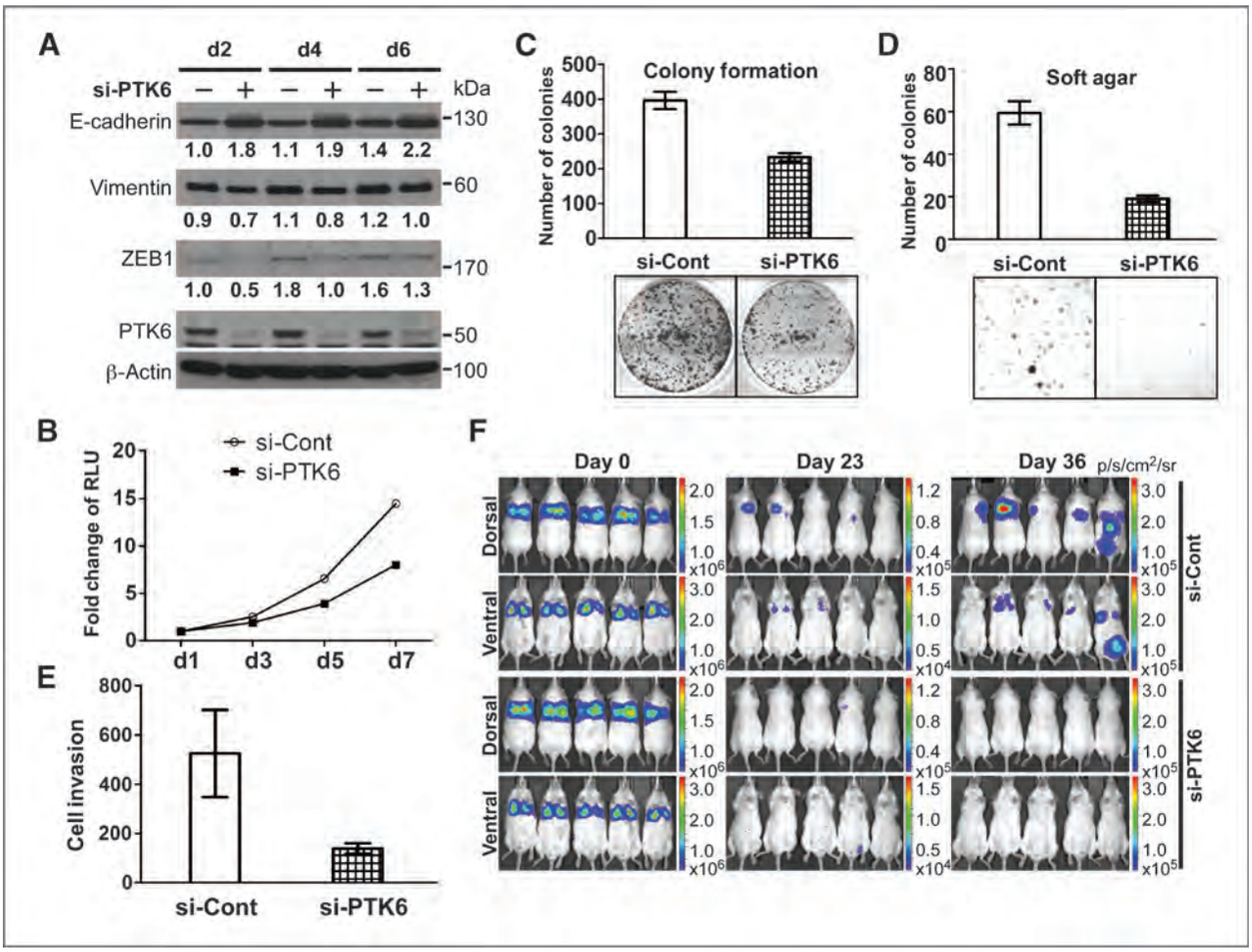

Figure 5. PC3 cells are less tumorigenic and invasive after knockdown of PTK6. A, E-cadherin is increased upon knockdown of PTK6 in PC3 cells. PC3 cells were transfected with PTK6 siRNAs or control siRNAs for 2, 4, or 6 days. Total cell lysates were analyzed by immunoblotting with anti-E-cadherin, ZEB1, PTK6, and β-actin antibodies. Relative levels of E-cadherin, vimentin, and ZEB1 expression normalized to actin levels are indicated below the blots. B, a growth curve of PC3 cells transfected with PTK6 siRNAs or control siRNAs shows decreased proliferation from day 1 to 7 after PTK6 knockdown. Relative light units (RLU) were measured by CellTiter-Glo Luminescent Cell Viability Assay. C, the number of colonies that form on plates 2 weeks postplating is decreased upon PTK6 knockdown. Corresponding images are shown below the graph. D, the number of colonies that form in soft agar 3 weeks postplating is decreased upon PTK6 knockdown. Representative images are shown. E, cell invasion is impaired upon PTK6 knockdown in Matrigel invasion chamber assays. F, knockdown of PTK6 in PC3 cells largely reduces metastases in SCID mice. PC3 cells stably expressing luciferase were transfected with PTK6 siRNA or control siRNA twice before injection. Mice were monitored under IVIS spectrum imaging system every week.

cancer samples and patient information was extracted from the Oncomine database (20). Patients were categorized into *PTK6* high, *PTK6* medium, and *PTK6* low groups according to their relative *PTK6* mRNA level. The Kaplan–Meier survival curve indicated that patients with higher *PTK6* mRNA expression have significantly poorer survival outcomes, whereas lower *PTK6* expression levels were associated with better overall survival ($P < 0.005$; Fig. 7A). Analysis of another dataset containing 140 prostate carcinoma samples with recurrence information was also extracted and analyzed using the Kaplan–Meier method (21). Higher *PTK6* expression was associated with earlier recurrence ($P < 0.05$; Fig. 7B). We have reported that *PTK6* expression is significantly increased in human metastatic prostate cancer (5). Analysis of the same dataset reveals decreased levels of E-cadherin in metastatic prostate cancer (Fig. 7C). Importantly, linear regression analyses show an inverse correlation of *PTK6* and E-cadherin mRNA in normal tissue and metastatic cancer groups, indicating one unit change of PTK6 can be used to predict change in E-cadherin levels (Fig. 7D). We also assessed activation of PTK6 in human prostate tumor tissues. PTK6 is highly activated at the plasma membrane of a group of tumor cells in a Gleason grade 4–5 prostate tumor (Fig. 7E, a and b), but not in two other Gleason grade 3 tumors (Fig. 7E, c and d). These data indicate that membrane-associated PTK6 activation is a marker for a subset of patients with prostate cancer and suggest that targeting PTK6 may have therapeutic benefits.

Discussion

A variety of studies indicate that PTK6 has context and condition-specific functions. PTK6 negatively regulates

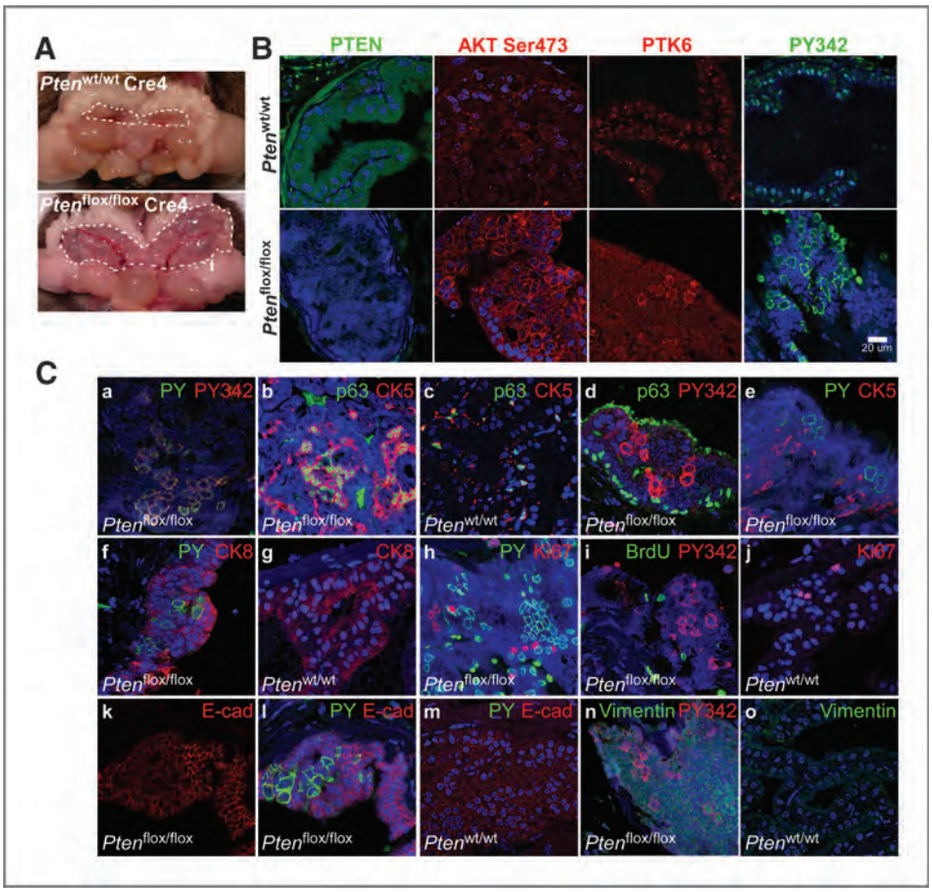

Figure 6. Aberrant activation of PTK6 is accompanied by deregulated E-cadherin at the plasma membrane in prostate tumor cells of PB-Cre4, *Pten*$^{flox/flox}$ mice. A, an enlarged anterior prostate was observed in PB-Cre4, *Pten*$^{flox/flox}$ mice at the age of 6 months. B, endogenous PTK6 is activated at the membrane in prostate tumor cells in a murine model (PB-Cre4, *Pten*$^{flox/flox}$). Immunohistochemistry was conducted with anti-PTEN, P-AKT (Ser473), PTK6, and P-PTK6 (Tyr342) antibodies, and samples were counterstained with DAPI (blue). Scale bar, 20 μm. C, prostate tumor cells with highly activated PTK6 at the plasma membrane undergo EMT. Immunohistochemistry was conducted with anti-phosphotyrosine, P-PTK6 (PY342), CK5, CK8, p63, BrdUrd (BrdU), Ki67, E-cadherin, and vimentin antibodies, and samples were counterstained with DAPI (blue).

proliferation, promotes differentiation, and mediates apoptosis in normal cells of the intestinal tract and skin, whereas it promotes proliferation, migration, and survival in breast, colon, ovarian, and prostate tumor cells (reviewed in refs. 8, 9, 26). Differences in PTK6 expression, activation, and intracellular localization, as well as expression of distinct sets of substrates and associated proteins in different cell types, would facilitate activation of distinct signaling pathways in normal and cancer cells. In normal cells, PTK6 is induced and activated in response to differentiation (27, 28) or stress such as DNA-damage (29, 30). On the other hand, the expression of PTK6 is significantly induced in various cancer cells, including breast cancer and prostate cancer (5, 31), where high levels of PTK6 predict poor prognosis in human patients (Fig. 7; ref. 32).

Our data are the first to show that activation of PTK6 at the membrane can positively contribute to the EMT. *In vivo* studies show that endogenous mouse PTK6 protein is active at the membrane in the *Pten* null prostate, and this correlates with reduced E-cadherin expression. In addition, we found that PTK6 is activated at the membrane in invasive human tumor samples. Expression of membrane-targeted PTK6 in PC3 cells led to repression of E-cadherin expression, a more mesenchymal phenotype, as well as increased tumorigenicity and metastases in xenograft models, further supporting a direct role for PTK6 in promoting the EMT. Recently, knockdown of PTK6 in a subline of human MCF-7 breast cancer cells engineered to overexpress HER2, led to increased E-cadherin and decreased mesenchymal marker expression, suggesting PTK6 also regulates the EMT in other cancers (33).

Membrane association of SRC kinases through amino-terminal lipid modification is critical for them to be able to transform cells (34). We have shown that even though PTK6 is not myristoylated/palmitoylated, the active endogenous protein can be found at the membrane (5, 11). Previously, we reported that membrane-targeted PTK6 has transforming potential, whereas nuclear PTK6 is growth inhibiting (4, 7). We showed that membrane-targeted PTK6 transforms mouse embryonic fibroblasts lacking the SRC-family members SRC, YES, and FYN (11). We detected nuclear localization of endogenous PTK6 in normal prostates, and relocalization of PTK6 to the cytoplasm and membrane in prostate tumors (Fig. 6; ref. 6). Activation and translocation of PTK6 in prostate cancer could lead to phosphorylation and activation of non-nuclear substrates to which it does not normally have access. Mechanisms regulating PTK6 intracellular shuttling are not well understood, but may be mediated through protein–protein interactions that could be modulated by expression of different PTK6 isoforms encoded by differentially spliced mRNAs (35).

PTK6 participates in several signaling pathways associated with cell migration, survival, and metastasis (reviewed in

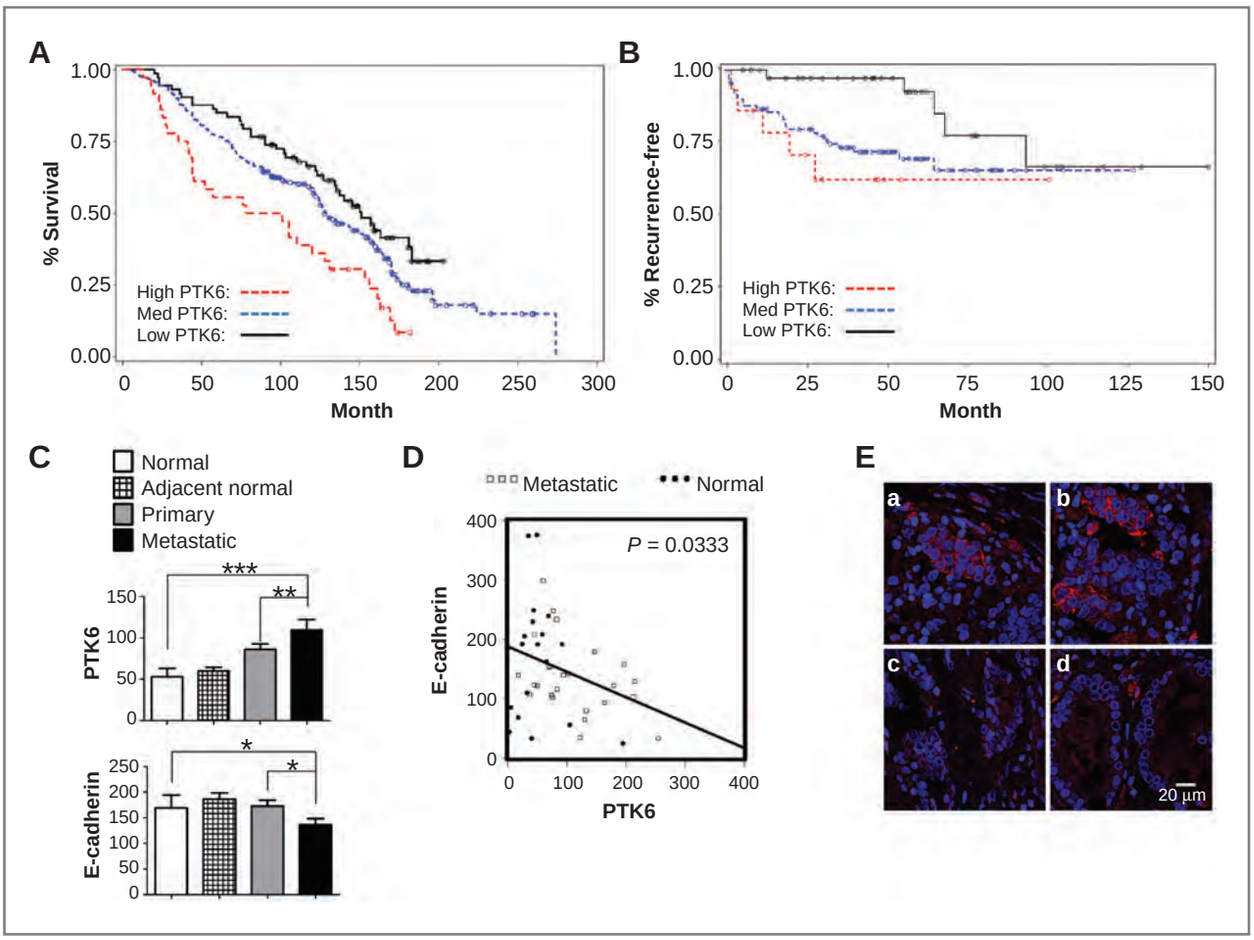

Figure 7. High levels of PTK6 predict poor prognosis of patients with prostate cancer. A, Kaplan–Meier survival curves of patients with low, medium, and high *PTK6* mRNA expression levels exhibit a significant difference in survival ($n = 36$ for high *PTK6*; $n = 254$ for medium *PTK6*; $n = 73$ for low *PTK6*; log-rank test $P < 0.005$; Wilcoxon test $P < 0.005$). B, Kaplan–Meier curves for the recurrence-free proportion of patients with low, medium, and high *PTK6* mRNA expression ($n = 14$ for high *PTK6*; $n = 88$ for medium *PTK6*; $n = 38$ for low *PTK6*; log-rank test $P < 0.05$; Wilcoxon test $P < 0.01$). C, increased levels of *PTK6* mRNA and decreased E-cadherin expression were detected in metastatic prostate cancer samples by analyzing the NCBI human genome microarray dataset GDS2545. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. D, *PTK6* expression is inversely correlated with levels of E-cadherin expression in normal tissue and metastatic cancer samples (dataset GDS2545) in a linear regression model. E, active PTK6 was detected at the plasma membrane of tumor cells in human prostate cancer samples (a and b, Gleason grade 4–5; c and d, Gleason grade 3). Immunohistochemistry was conducted using human prostate tumor tissue with anti-P-PTK6 (PY342) antibodies, and samples were counterstained with DAPI (blue). Scale bar, 20 µm.

refs. 26, 36). It regulates signaling by ERBB receptors (37, 38), the hepatocyte growth factor (HGF) receptor MET (39), and IGF-I (32, 40). Its substrates include Paxillin (41), AKT (10), EGFR (42), p130CAS (5), and FAK (11). PTK6 also regulates p190RhoGAP (43) and ERK5 (44) activity. PTK6-mediated deregulation of E-cadherin could involve several PTK6 downstream players, including AKT, p130CAS, FAK, and ERK5. AKT signaling promotes the EMT in different cancer cell lines (23, 45, 46). PTK6 confers resistance to anoikis, a hallmark of the EMT (reviewed in ref. 47), which may occur through both direct and indirect activation of AKT (5, 10, 11). AKT is a direct substrate of PTK6 and it is also activated downstream of the PTK6 substrates p130CAS (5) and FAK (11). Here, we show that Palm-PTK6 promotes AKT activation and regulation of its downstream targets, including GSK3β and the E-cadherin repressor SLUG (Fig. 3), and this contributes in part to PTK6-mediated regulation of the EMT. Knockdown of the PTK6 substrate p130CAS impairs AKT activation (5), and partially rescues E-cadherin downregulation induced by Palm-PTK6-YF (Fig. 3D). Previously, we have shown that ERK5 plays an important signaling role downstream of p130CAS in cells expressing membrane-targeted active PTK6 (5). ERK5 has been implicated in breast cancer cell metastasis (48), and is required for HGF-induced cell migration in breast cancer cells (39).

In prostate cancer, decreased levels of E-cadherin are associated with high prostate tumor grade and poor prognosis. Patients with normal E-cadherin expression have a significantly higher overall survival rate than patients with low expression (49, 50). Here, we show that PTK6 is aberrantly expressed and activated in prostate tumor cells in some patients, and its levels are inversely correlated with E-cadherin expression in metastatic prostate cancer (Fig. 7). Targeting PTK6 using siRNAs

dramatically reduces the metastatic potential of human prostate cancer cells in a mouse xenograft model (Fig. 5F). Our findings suggest that PTK6 is a novel gene marker in categorizing prostate cancer patient groups, and a potential gene target for personalized medicine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Zheng, Z. Wang, A.L. Tyner
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Correction

Cancer Research

Correction: PTK6 Activation at the Membrane Regulates Epithelial–Mesenchymal Transition in Prostate Cancer

In this article (Cancer Res 2013;73:5426–37), which was published in the September 1, 2013, issue of *Cancer Research* (1), the citation and order of some of the References were incorrect due to a production error. These errors have been corrected in the online version of the article, which now no longer matches the print version.

Reference

1. Zheng Y, Wang Z, Bie W, Brauer PM, Perez White BE, Li J, et al. PTK6 activation at the membrane regulates epithelial–mesenchymal transition in prostate cancer. Cancer Res 2013;73:5426–37.

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REVIEW

Context-specific protein tyrosine kinase 6 (PTK6) signalling in prostate cancer

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ABSTRACT

Background Protein tyrosine kinase 6 (PTK6) is an intracellular tyrosine kinase that is distantly related to SRC family kinases. PTK6 is nuclear in normal prostate epithelia, but nuclear localization is lost in prostate tumours. Increased expression of PTK6 is detected in human prostate cancer, especially at metastatic stages, and in other types of cancers, including breast, colon, head and neck cancers, and serous carcinoma of the ovary.

Materials and methods Potential novel substrates of PTK6 identified by mass spectrometry were validated *in vitro*. The significance of PTK6-induced phosphorylation of these substrates was addressed using human prostate cell lines by knockdown of endogenous PTK6 or overexpression of targeted PTK6 to different intracellular compartments.

Results We identified AKT, p130CAS and focal adhesion kinase (FAK) as novel PTK6 substrates and demonstrated their roles in promoting cell proliferation, migration and resistance to anoikis. In prostate cancer cells, active PTK6 is primarily associated with membrane compartments, although the majority of total PTK6 is localized within the cytoplasm. Ectopic expression of membrane-targeted PTK6 transforms immortalized fibroblasts. Knockdown of endogenous cytoplasmic PTK6 in PC3 prostate cancer cells impairs proliferation, migration and anoikis resistance. However, re-introduction of PTK6 into the nucleus significantly decreases cell proliferation, suggesting context-specific functions for nuclear PTK6.

Conclusions In human prostate cancer, elevated PTK6 expression, translocation of PTK6 from the nucleus to the cytoplasm and its activation at the plasma membrane contribute to increased phosphorylation and activation of its substrates such as AKT, p130CAS and FAK, thereby promoting prostate cancer progression.

Keywords AKT, BRK, ERK5, FAK, p130CAS, PTK6.

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Introduction

Prostate cancer is the most common form of cancer, other than skin cancer, in American men. About one out of six men will be diagnosed with prostate cancer during their lifetime. Although prostate cancer has a relatively low mortality rate, it remains the second leading cause of cancer-related deaths in American men [1]. The major cause of death is metastases resulting from lymphatic, blood or contiguous local spread. Unfortunately, we still lack effective means to treat metastatic prostate cancer, and the use of tyrosine kinase inhibitors is being explored as a treatment option [2,3].

Roles for nonreceptor tyrosine kinases in prostate cancer have been previously reviewed [4]. SRC, FAK, JAK1/2 and ETK/BMX play indispensable roles in different aspects of prostate cancer including proliferation, migration, apoptosis and metastasis [4]. During the last few years, we have made substantial progress in understanding functions of the intracellular tyrosine kinase, protein tyrosine kinase 6 (PTK6), and its potential contributions to prostate cancer, and these new findings are reviewed here [5–9].

Protein tyrosine kinase 6 belongs to the PTK6 family of intracellular nonreceptor tyrosine kinases, which includes Fyn-related kinase (FRK, also known as RAK, BSK, Iyk and Gtk) and **S**RC-**R**elated kinase lacking C-terminal regulatory tyrosine and N-terminal Myristoylation Sites (SRMS). These proteins are structurally similar to the SRC family kinases, consisting of Src-homology-3 (SH3) and SH2 domains followed by a tyrosine kinase catalytic domain. However, they lack an SH4 domain, which facilitates lipid modification and membrane association, and they exhibit flexibility in intracellular localization (reviewed in [10]). PTK6 family members share a highly conserved gene structure that is distinct from other intracellular tyrosine kinase families, including the SRC family [11,12].

Human PTK6 was identified in cultured human melanocytes [13] and breast tumour cells [14] and has been commonly referred to as BReast tumour Kinase (BRK). Its mouse orthologue was cloned from normal small intestinal epithelial cell RNA in a screen for factors that regulate epithelial cell turnover, and was thus given the name SRC-related intestinal kinase (Sik) [15,16].

Different roles for PTK6 in normal tissues and cancer

In normal tissues, expression of PTK6 is highest in the nondividing, differentiated epithelial cells of the gastrointestinal tract [16,17]. PTK6 is also detected in other differentiated epithelial cells including those of the prostate [18], oral cavity [19] and skin [16,20]. A variety of studies suggest that PTK6 negatively regulates proliferation and promotes the differentiation of epithelial cells. In the cultured human keratinocyte cell line HaCaT and embryonic mouse keratinocyte cell line EMK, addition of calcium promotes differentiation, which is accompanied by increased PTK6 expression and activation, and elevated levels of the epidermal differentiation markers, keratin-10 in HaCaT cells and filaggrin in EMK cells [20,21]. Studies in a *Ptk6*-deficient mouse model demonstrated roles for PTK6 in promoting cell cycle exit and differentiation in the normal intestinal epithelium *in vivo*. Increased growth of small intestinal villi was accompanied by an expanded zone of proliferation and delayed enterocyte differentiation in *Ptk6*-/- mice [22].

Protein tyrosine kinase 6 also plays important roles in regulating the survival of normal cells in response to a variety of apoptotic stimuli. PTK6 sensitized immortalized nontransformed Rat1A cells to apoptosis induced by serum deprivation and UV irradiation [23]. Further *in vivo* studies revealed that PTK6 expression is induced in small intestinal crypt epithelial cells by γ-radiation, where it appears to promote DNA damage –induced apoptosis by inhibiting prosurvival signalling including AKT and ERK1/2 [24]. In the colon, induction of PTK6 in crypt base epithelial cells following administration of the carcinogen azoxymethane was also positively correlated with apoptosis [25].

Although PTK6 is not expressed in the normal human mammary gland, it is aberrantly expressed in a high percentage of breast tumours [26,27]. Elevated expression of PTK6 has also been detected in other types of cancer including colon [17], prostate [8], head and neck cancer [28], serous carcinoma of ovary [29], lung [30] and thyroid cancer [31]. Recently, however, expression of PTK6 transcripts was found to be downregulated in oesophageal squamous carcinomas as a consequence of epigenetic modification, and PTK6 appears to have tumour suppressor activities in this type of cancer [32].

Several studies have demonstrated oncogenic roles for PTK6 using established cancer cell lines and animal models. PTK6 promotes breast cancer cell proliferation through phosphorylating and activating its substrates STAT3 [33] and STAT5b [34], and this process may be facilitated by STAP2 [35,36], a scaffold protein that is also a PTK6 substrate. Interestingly, the suppressor of cytokine signalling 3 (SOCS3) has been identified as an inhibitor of PTK6 [37]. PTK6 phosphorylates paxillin and p190RhoGAP-A to promote EGF-dependent cell migration and invasion [38,39]. PTK6 was identified from an siRNA screen as a critical regulator of IGF-1 mediated anchorage-independent survival of breast and ovarian tumour cells [40]. In addition, positive crosstalk between PTK6 and membrane receptors such as EGFR, HER2/Neu or MET has also been reported (reviewed in [10,41,42,43]). Recently, PTK6 was shown to sustain EGFR signalling by directly phosphorylating EGFR and inhibiting its downregulation [44].

In a WAP-driven PTK6 transgenic FVB/N mouse model, delayed involution of the mammary gland might be caused by activation of a p38 MAPK prosurvival signalling pathway, and aged mice developed infrequent tumours with reduced latency compared with wild-type mice [27]. In the AOM/DSS (azoxymethane/dextran sodium sulphate) murine colon cancer model, disruption of *Ptk6* impaired colon tumorigenesis, probably due to significantly reduced STAT3 activation [25].

While most available data suggest that PTK6 overexpression in cancer is oncogenic, some studies have correlated PTK6 expression with increased survival [45,46] and tumour suppression [32]. This suggests that PTK6 could have more complex roles in cancer, which may be related to tumour heterogeneity and its intracellular localization and access to specific substrates. The 'pro'-oncogenic role of PTK6 in most tumours and the 'anti'tumorigenic role of PTK6 in normal cells might be achieved by activation of distinct signalling pathways due to cell/tissue type, PTK6 expression levels, alterations in intracellular location and different environmental stimuli. Oncogenic functions of PTK6 are enhanced when the protein is targeted to the plasma membrane in HEK-293 cells [47]. Our group first proposed that intracellular localization of PTK6 will have an impact on its cellular functions [18,48], and discovered that nuclear-targeted PTK6 negatively regulates, whereas membrane-targeted active PTK6 enhances endogenous β-catenin/TCF transcriptional activity in SW620 colon cancer cells [49].

Aberrant expression, localization and activation of PTK6 in human prostate cancer

To understand the role of PTK6 in prostate cancer, we analysed the NCBI human genome microarray data set GDS2545, which contains 171 samples [50]. *PTK6* mRNA levels were

significantly higher in prostate tumour samples, especially in metastatic prostate tumour samples compared with normal prostate tissue and normal tissue adjacent to the tumour, indicating an oncogenic role for PTK6 in prostate tumorigenesis and metastasis [8].

Protein tyrosine kinase 6 is primarily localized within the nuclei of normal human prostate epithelial cells, but it is localized to the cytoplasm and at the membrane in poorly differentiated prostate tumours [18]. In the established human prostate cancer cell lines PC3 (Androgen Receptor/AR negative) and LNCaP (AR positive), the majority of PTK6 is localized within the cytoplasm, although nuclear PTK6 can also be detected by immunostaining in the more differentiated LNCaP cancer cell line [8,18]. Prostate cancer cells provide a suitable system to investigate the biological significance of PTK6 translocation.

Interestingly, although only a small fraction of total PTK6 was localized in the membrane compartment in PC3 cells, membrane-associated PTK6 was highly phosphorylated at tyrosine residue 342, which is a marker for its kinase activation. In contrast, the more abundant pool of cytoplasmic PTK6 was not phosphorylated at this tyrosine residue. Targeting exogenous PTK6 with a mutation of its inhibitory tyrosine residue 447 (Y-F) to membrane compartments by addition of a palmitoylation/myristoylation consensus sequence (Palm) at the amino-terminus largely increases the active pool of PTK6 in PC3 cells [8]. In addition, compared with untargeted PTK6-YF, Palm-PTK6-YF (membrane-targeted) showed substantially higher activity in SYF cells *(Src-/-, Yes-/-, Fyn-/-* mouse embryonic fibroblasts) [8,9]. These data indicate that membrane localization of PTK6 is critical for its activation and support the hypothesis that translocation of PTK6 from nucleus to cytoplasm/membrane in prostate cancer could promote its activation and access to different substrates. Understanding the regulation of this relocalization and activation of PTK6 could shed light on novel mechanisms that drive prostate tumorigenesis and metastasis.

Membrane-associated active PTK6 promotes prostate cancer cell migration by phosphorylating p130CAS and activating ERK5

To further understand PTK6 signalling mechanisms and identify new substrates, proteins whose phosphorylation was increased upon ectopic expression of active PTK6 in human cells were identified using liquid chromatography coupled with tandem mass spectrometry [9]. Along with the previously identified PTK6 substrates Sam68, paxillin and PSF, we identified several novel candidates, including p130 CRK-associated substrate (p130CAS) [8] and focal adhesion kinase (FAK) [9], and further demonstrated that PTK6 directly phosphorylates them *in vitro*. p130CAS is a scaffolding protein that includes a domain containing 15 repeats of a YXXP motif that can be targeted by SRC family kinases [51]. Tandem mass spectrometry revealed 11 tyrosine residues within the substrate domain that can be targeted by PTK6 *in vitro* [8]. FAK is also a multidomain protein that can be phosphorylated by SRC family kinases at several tyrosine residues including 576/577, 861 and 925. Phosphorylation of tyrosine residues 576 and 577 is crucial in achieving maximum kinase activity, while phosphorylated tyrosine residue 925 is believed to be a high-affinity Grb2 binding site (reviewed in [52]). Tandem mass spectrometry analyses showed that PTK6 phosphorylates FAK at tyrosine residue 861 *in vitro*, although the biological significance of the phosphorylation on this residue is not clear [9].

Both p130CAS and FAK are concentrated at focal adhesions [53,54]. Following integrin clustering, FAK phosphorylates p130CAS at its C-terminal Y_{664}DYVHL motif and then SRC phosphorylates p130CAS at several tyrosine residues within its substrate domain, which provide binding sites for the adaptor protein CRK, leading to the activation of the small GTPase RAC that is able to induce membrane ruffling, cytoskeleton remodelling and cell migration [55,56]. Expression of membrane-targeted active PTK6 in PC3 cells induced membrane ruffling and formation of specific structures called peripheral adhesion complexes, which have been observed in active SRC-expressing KM12C colon cancer cells [8,57]. Both the kinase activity and membrane localization of PTK6 are necessary for the formation of peripheral adhesion complexes. Tyrosine phosphorylation of p130CAS and FAK are both induced and enriched in these structures [8]. Interestingly, the formation of peripheral adhesion complexes induced by PTK6 is dependent on p130CAS but not FAK, as knockdown of p130CAS impaired their formation, but knockdown of FAK did not. It is possible that PTK6, unlike SRC, does not rely on FAK to initiate the phosphorylation at the YDYVHL motif of p130CAS [8].

We also demonstrated that ERK5 but not ERK1/2 is enriched in peripheral adhesion complexes induced by membrane-targeted active PTK6. Knockdown of p130CAS impaired ERK5 activation in response to serum stimulation, indicating that ERK5 is activated downstream of p130CAS [8]. p130CAS may serve as a scaffold protein that provides multiple phosphorylated tyrosine residues as binding sites for downstream interacting partners, to convey the Palm-PTK6-YF induced oncogenic signalling. It has been reported that PTK6 forms complexes with ERK5 in various human cells [58], but whether p130CAS and ERK5 are in the same complex is not known. As with other focal adhesion-like structures such as invadopodia and podosome, formation of peripheral adhesion complexes is accompanied by increased cell migration. This is dependent on p130CAS and ERK5, as knockdown of either protein impaired

the formation of peripheral adhesion complexes and cell migration [8].

Activation of PTK6 at the membrane protects cells from anoikis through activation of FAK and AKT survival signalling

Membrane-targeted active PTK6 is able to transform murine embryonic fibroblasts, even in the absence of the SRC family kinases Src, Yes and Fyn. One of the most striking features of Palm-PTK6-YF-transformed SYF cells is their ability to overcome anoikis and maintain proliferation under suspended growth conditions. Palm-PTK6-YF-mediated FAK phosphorylation and the subsequent activation of AKT survival signalling contribute to this process [9].

In the absence of FAK, Palm-PTK6-YF was still able to protect *Fak*-/- MEFs from anoikis, indicating it has the ability to activate survival signalling independent of FAK. However, stable co-expression of FAK and Palm-PTK6-YF in *Fak* -/- MEFs synergistically activated AKT survival signalling and protected cells against anoikis. These data demonstrated that, while not essential, FAK is involved in PTK6-meditated anoikis resistance. Interestingly, Palm-PTK6-YF failed to protect *Akt1*/2-/- MEFs from anoikis, suggesting AKT is a critical downstream player that mediates survival signalling induced by PTK6 or FAK activation [9].

PTK6 directly phosphorylates AKT at tyrosine residues and promotes its activation

AKT is a direct substrate of PTK6. Tandem mass spectrometry analysis revealed that tyrosine residues 215 and 326 of AKT can be phosphorylated by PTK6. Further point mutation studies demonstrated that tyrosine residues 315 and 326 of AKT are the primary targets of PTK6, residues that can also be phosphorylated by SRC [7,59]. Importantly, PTK6 induced the tyrosine phosphorylation of AKT in SYF cells, which lack endogenous Src, Yes and Fyn. In the presence of exogenous PTK6, SYF cells have increased levels of AKT activation (marked by phosphorylation of Threonine 308 and Serine 473) in response to physiological levels of EGF, which is accompanied by increased AKT tyrosine phosphorylation. SYF cells expressing active PTK6, but not kinase-dead PTK6, showed increased cell proliferation [7].

Knockdown of endogenous PTK6 impairs tumorigenicity of prostate cancer cells

Protein tyrosine kinase 6 is primarily localized within the cytoplasm in the highly tumorigenic PC3 prostate cancer cell line [8]. Stable knockdown of PTK6 in PC3 cells using two different shRNA constructs impaired cell proliferation and colony formation [5]. PTK6 also plays an important role in cell migration, as transient knockdown of PTK6 using siRNAs largely decreased cell migration, which was accompanied by decreased p130CAS phosphorylation and ERK5 activation [8]. Moreover, PTK6 is primarily responsible for the anoikis resistance of metastatic prostate cancer PC3 cells, which are able to maintain an ~80% survival rate under suspended growth conditions for 8 days [9]. Knockdown of PTK6 in PC3 cells induced apoptosis under suspended growth conditions, which was accompanied by reduced FAK and AKT activation [9]. Interestingly, while PTK6 and SRC share several common substrates, knockdown of SRC in PC3 cells had only a small impact on the anoikis resistance of PC3 cells, and knockdown of both SRC and PTK6 did not have a synergistic effect, indicating PTK6 is the key player in protecting prostate cancer cells from anoikis [9]. These data demonstrate the oncogenic role of PTK6 in various aspects of tumorigenicity, including growth, survival and cell migration, and suggest that targeting PTK6 might be beneficial in treating human prostate cancer.

Introducing PTK6 into the nucleus of PC3 cells negatively regulates growth

Knockdown of PTK6 in PC3 cells led to growth inhibition, supporting a growth-promoting role for endogenous cytoplasmic PTK6 in this prostate cancer cell line. However, re-introduction of ectopic PTK6 into the PC3 cell nucleus by addition of a SV40 nuclear localization signal to the amino-terminus of PTK6 also led to growth inhibition that was dependent upon PTK6 activity [5]. These data suggest that nuclear PTK6 is important for maintaining proper growth regulation in the normal prostate.

Different functions for PTK6 within the nucleus and at the membrane could be explained by its access to unique sets of substrates and interacting proteins in the different compartments. Sam68, a known PTK6 substrate [60], is a multifunctional KH domain–containing protein with several proline-rich motifs that has context-specific RNA-binding and adaptor protein functions [61,62]. It is largely nuclear in normal prostate and prostate tumours [18], and is phosphorylated on tyrosine residues by nuclear-targeted PTK6 [5]. Sam68 has been implicated in development of prostate cancer. Expression of Sam68 was increased in 35% of prostate cancer samples examined, and knockdown of Sam68 led to reduced proliferation of cultured prostate cancer cells [63]. Nuclear PTK6 in the normal prostate may inhibit growth by increasing tyrosine phosphorylation of Sam68 and related RNA-binding proteins, leading to inhibition of their RNA-binding activities and affecting different aspects of RNA metabolism, including RNA stability, translation and transport [5,60]. Following its relocalization to the cytoplasm/

membrane in prostate cancer, PTK6 would no longer have access to nuclear Sam68 (Fig. 1).

Protein tyrosine kinase 6 can also interact with and phosphorylate β-catenin on multiple tyrosine residues [49]. β-catenin, which has distinct membrane and nuclear functions, regulates both cell adhesion and transcription [64]. It is a key component of the WNT signalling pathway that is involved in promoting prostate cancer progression [65]. Nuclear-targeted PTK6 was shown to inhibit β-catenin-/TCF-regulated transcription in colon cancer cells [49]. In contrast, expression of membrane-targeted active PTK6 led to increased β-catenin-/TCF-regulated transcription [49]. At the membrane, PTK6 can phosphorylate β-catenin on tyrosine residue 142, inhibiting its membrane and promoting its nuclear functions [49] (Fig. 1). Differential regulation of β-catenin would provide another mechanism by which nuclear PTK6 could inhibit, while membrane-associated PTK6 could promote prostate epithelial cell proliferation, and requires further investigation.

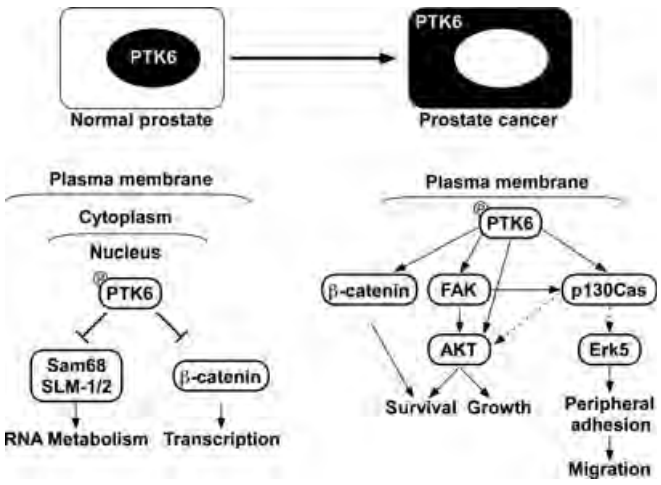

Figure 1 Distinct functions for protein tyrosine kinase (PTK6) in the nucleus and at the plasma membrane. In normal prostate epithelial cells, total and active PTK6 is enriched in the nucleus where it has access to specific nuclear substrates and interacting proteins. These include the RNA-binding proteins Sam68 [60] and SLM1 and SLM2 [48], as well as β-catenin [49]. PTK6 inhibits the RNA-binding abilities of Sam68 [60] and the transcriptional activities of β-catenin [49]. Relocalization of PTK6 from nucleus to cytoplasm in prostate cancer facilitates its activation at the membrane. Active PTK6 can then phosphorylate and activate its cytoplasmic and membrane substrates including p130CAS [8], FAK [9], AKT [7] and β-catenin [49] to promote cancer cell proliferation, survival and migration.

Potential mechanisms underlying PTK6 translocation: a role for the alternative *PTK6* transcript ALT-PTK6?

Protein tyrosine kinase 6 lacks both membrane and nuclear targeting signals, but it is largely nuclear in normal prostate epithelium and cytoplasm/membrane associated in prostate cancer [18]. A particularly interesting question that still needs to be addressed is, 'How does PTK6 translocate from the nucleus to the cytoplasm during the development/progression of prostate cancer?' We determined that translocation is not due to aberrant nuclear export mediated by Crm-1/exportin-1, because inhibiting Crm-1/exportin-1 using leptomycin B did not result in nuclear accumulation of PTK6 in PC3 cells. Moreover, overexpression of Sam68, a nuclear binding partner of PTK6, was not sufficient to bring PTK6 into the nucleus [5].

Interestingly, expression of ALT-PTK6, which is encoded by an alternatively spliced PTK6 transcript, is able to affect the intracellular localization of exogenous PTK6 in HEK-293 cells. ALT-PTK6 contains the intact SH3 domain of PTK6 and could compete with PTK6 for binding of specific cytoplasmic substrates and binding partners. In cotransfection studies, increased ectopic expression of ALT-PTK6 led to increased nuclear localization of full-length active PTK6 and inhibition of β-catenin/TCF transcription. ALT-PTK6 may compete with full-length PTK6 for binding to a cytoplasmic retention factor that has yet to be identified, allowing PTK6 to re-enter the nucleus and inhibit β-catenin-induced transcription [6]. ALT-PTK6 expression is decreased in human prostate cancer samples, which may enhance the cytoplasmic retention and oncogenic signalling of PTK6 [6].

Conclusions

Functions of PTK6 are highly context dependent and distinct in normal tissues and cancer. Increased growth, impaired enterocyte differentiation [22] and impaired DNA damage–induced apoptosis [24] led to the hypothesis that PTK6 might act as a tumour suppressor in the intestine. Surprisingly, *Ptk6* null mice were resistant to AOM/DSS-induced colon tumorigenesis refuting this notion [25]. However, recent studies suggest that PTK6 functions as a tumour suppressor in oesophageal squamous cell cancers [32]. In contrast, oncogenic roles for PTK6, particularly in breast cancers (reviewed in [10,41]) are supported by a large body of data. Identified inhibitors of PTK6 that may have therapeutic potential under the appropriate conditions include the BRAF inhibitor PLX4032 [66], Geldanamycin, an Hsp90 inhibitor [67], SOCS3 [37] and a series of substituted imidazo[1,2-a]pyrazin-8-amines [68].

The prostate provides a unique model where the importance of PTK6 expression levels and intracellular localization can be

addressed. Increased expression, altered intracellular localization and activation at the plasma membrane are characteristics of PTK6 in prostate cancer. These characteristics may promote its access to distinct cytoplasm- and membrane-associated substrates and interacting proteins including AKT [7], β-catenin [49], p130CAS [8], and FAK [9]. PTK6-mediated phosphorylation and/or association with these proteins could lead to the activation of oncogenic signalling pathways that are involved in regulating cell growth, survival and migration, therefore promoting prostate cancer progression. The relocalization of PTK6 and its activation in different cellular compartments could serve as a marker for cancer staging and prognosis. Recently, we demonstrated that targeting PTK6 enhances the response of colon cancer cells to chemotherapeutic agents [69]. Studies in prostate cancer cells suggest that targeting PTK6 in prostate cancer may also have significant therapeutic benefits.

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